NOVEL TOPOISOMERASE IV, CORRESPONDING NUCLEOTIDE SEQUENCES AND USES THEREOF

The present invention relates to a novel topoisomerase IV, the nucleotide sequences encoding this enzyme, their corresponding vectors and the use of this enzyme for screening biologically active products.

Topoisomerases are enzymes capable of modifying the topological configuration of DNA rings, of making knots therein or of interlacing separated rings. They are thus involved in the replication, transcription and recombination of the entire genetic information (Wang et al., 1990). The mechanism of all these topological conversions is the same: the ring is opened so that a segment of DNA passes through the gap before the ends are rejoined. Two types of topoisomerase are involved in these conversions: type I topoisomerases which cut a single DNA strand and type II topoisomerases which cut both strands simultaneously.

Up until now, two type II bacterial topoisomerases have been identified and studied more particularly: gyrase from Escherichia coli (Gellert et al., 1976), and more recently, DNA topoisomerase IV from E.coli (Kato et al., 1990).

Gyras is a $\alpha_2\beta_2$ tetramer whose α or GyrA and β or GyrB subunits are encod d by the gyrA and gyrB genes resp ctively. Bacterial gyrases ar th only

known topoisomeras s capabl of supercoiling r lax d DNA rings in the presence of ATP.

As regards more particularly DNA topoisomerase IV from E.coli, it relaxes supercoiled plasmid DNA, unknots T4 phage DNA and unwinds (or decatenates) kinetoplast DNA (Kato et al., 1992; Peng et al., 1993). The sequence of its corresponding genes, parC and parE from E.coli, has made it possible to demonstrate regions of high similarity between the subunits of gyrase and those of this topoisomerase IV, ParC with GyrA (35.6% over the entire sequence) and ParE with GyrB (40.1% over the entire sequence) respectively (Kato et al., 1990).

E.coli gyrase has also been identified as being a primary target of fluoroquinolones (Hooper et al., 1993). It has thus been demonstrated that E.coli strains mutated at the level of the Ser83 residue in the GyrA subunit have a high resistance to fluoroquinolones (Maxwell, 1992). Fluoroquinolones bind less to DNA-mutated gyrase complexes than to DNA-wild-type gyrase complexes. Indeed, other point mutations, mapped in the region between residues 67 and 106 of GyrA, lead to strains resistant to fluoroquinolones. This region is called QRDR (Yoshida et al., 1990; Cullen et al., 1989). Similar r sults have been published with strains of Staphylococcus aureus r sistant to fluoroquinolones (Goswitz et al., 1992; Sre dharan t al., 1990). Gyrase is therefor nowadays

recogniz d as being th primary target of quinolon s. However, a clinical strain of Staphylococcus aureus, not containing any mutation in the QRDR region of GyrA, has also been described as resistant to fluoroquinolones (Sreedharan et al., 1991).

Nowadays, this phenomenon of resistance developed by Staphylococcus aureus bacteria towards antibiotics and more particularly towards fluoroquinolones is being increasingly encountered at the therapeutic level. It would be particularly important to be able to lift this resistance and this involves a characterization of all the parameters which are associated with it.

The main objective of the present invention is precisely the identification, sequencing and characterization of nucleic sequences encoding subunits of a novel topoisomerase, topoisomerase IV of Staphylococcus aureus, composed of two subunits, GrlA and GrlB.

Unexpectedly, the applicant has found that the primary target of the fluoroquinolones in S. aureus is a topoisomerase IV and not gyrase. It has thus demonstrated that clinical strains of S. aureus, in which the QRDR region of the GyrA subunit of gyrase is identical to th wild-type sequence, d v lop n v rth less a resistance to fluoroquinolones becaus of a mutation which th y possess in th r gion of the GrlA subunit of topoisomerase IV, homologous to the

QRDR region.

The first subject of the present invention is a nucleotide sequence encoding at least one subunit of topoisomerase IV of Staphylococcus aureus.

The present invention describes in particular the isolation and the characterization of the grlA and grlB genes. These genes have been cloned, sequenced and expressed in E. coli, and their enzymatic activity has been characterized. They were isolated from a Staphylococcus aureus genomic DNA library. From the grlAB nucleic sequence (SEQ ID No. 1), two open frames, corresponding to the grlB and grlA genes respectively, have been identified. The grlA and grlB genes have been sequenced in SEQ ID No. 2 and SEQ ID No. 3 respectively.

Preferably, the subject of the present invention is a nucleotide sequence chosen from:

(a) all or part of the grlA (SEQ ID No. 2) or grlB (SEQ ID No. 3) genes,

(b) the sequences hybridizing with all or part of the (a) genes and encoding a subunit of a topoisomerase IV, and

(c) the sequences derived from the (a) and (b) sequences because of the degeneracy of the genetic code.

It is clear that from the genes identified in the present application, it is possible, by hybridization, to directly clone other genes encoding a

subunit of topoisomerase IV of bacteria close to *S. aureus* such as for example Streptococci and Enterococci. It is thus possible to clone this type of gene using, as probe, the genes *grlA*, *grlB* or fragments thereof. Likewise, the cloning of these genes may be carried out using degenerate oligonucleotides derived from sequences of the *grlA* or *grlB* genes or fragments thereof.

For the purposes of the present invention, derivative is understood to mean any sequence obtained by one or more modifications and encoding a product conserving at least one of the biological properties of the original protein. Modification should be understood to mean any mutation, substitution, deletion, addition or modification of a genetic and/or chemical nature. These modifications may be performed by techniques known to persons skilled in the art.

Among the preferred derivatives, there may be mentioned more particularly natural variants, molecules in which one or more residues have been substituted, derivatives obtained by deletion(s) of regions not or little involved in the interaction between the binding sites considered or expressing an undesirable activity, and derivatives having, compared with the native sequence, one or more additional residues.

Still more preferably, the subject of the invention is the nucleotide sequences represented by the *grlA* (SEQ ID No. 2) and *grlB* (SEQ ID No. 3) genes.

It also relates to any grlA g n having a mutation leading to a resistance to molecules of the quinolone and more particularly of the fluoroquinolone family. As a representative of these mutated genes, there may be mentioned more particularly the grlA gene having a base change from C to A at position 2270 of SEQ ID No. 2. The resulting gene is termed grlA $_{(C-2270A)}$. This mutation leads to substitution of the Ser-80 residue with Tyr in the GrlA protein. The resulting protein will be designated by GrlA $_{(Ser-80\ Tyr)}$.

Another subject of the present invention relates to a recombinant DNA comprising at least one nucleotide sequence encoding a subunit of topoisomerase IV of Staphylococcus aureus. More preferably, it is a recombinant DNA comprising at least one nucleotide sequence as defined above in (a), (b) and (c) and more particularly the gene grlA (SEQ ID No. 2) grlA $_{(C-2270A)}$ and/or the gene grlB (SEQ ID No. 3).

According to a preferred mode of the invention, the nucleotide sequences defined above form part of an expression vector which may be autonomously replicating or integrative.

Another subject of the invention relates to the polypeptides resulting from the expression of the nucl otid sequ nces as d fin d above. Mor particularly, the pr s nt invention relat s to th polyp ptides comprising all or part of the polyp ptid s GrlA (SEQ ID No. 2) or GrlB (SEQ ID No. 3) or of th ir

derivativ s. For th purposes of the pres nt inv ntion, the term derivative designates any molecule obtained by modification of the genetic and/or chemical nature of the peptide sequence. Modification of the genetic and/or chemical nature may be understood to mean any mutation, substitution, deletion, addition and/or modification of one or more residues. Such derivatives may be generated for different purposes, such as especially that of increasing the affinity of the peptide for its substrate(s), that of enhancing its production levels, that of increasing its resistance to proteases, that of increasing and/or of modifying its activity, or that of conferring new biological properties on it. Among the derivatives resulting from an addition, there may be mentioned, for example, the chimeric polypeptides containing an additional heterologous part attached to one end. The term derivative also comprises the polypeptides homologous to the polypeptides described in the present invention, derived from other cellular sources.

Preferably, they are the polypeptides GrlA (SEQ ID No. 2), GrlB (SEQ ID No. 3) and GrlA $_{(Ser\text{-}80Tyr)}$.

The subject of the invention is also any recombinant cell containing a nucleotide sequence, a recombinant DNA and/or a v ctor as defined abov . The recombinant cells according to the inv ntion may b both eukaryotic and prokaryotic cells. Among th suitabl eukaryotic c lls, there may be m ntion d

animal cells, y asts, or fungi. In particular, as regards yeasts, there may be mentioned yeasts of the genus Saccharomyces, Kluyveromyces, Pichia, Schwanniomyces or Hansenula. As regards animal cells, there may be mentioned COS, CHO and C127 cells, Xenopus eggs, and the like. Among the fungi, there may be mentioned more particularly Micromonospora, Aspergillus ssp. or Trichoderma ssp. Preferably, they are prokaryotic cells. In this case, the following bacteria may be more particularly used: Actinomycetes, Bacillus, and more preferably E. coli and Staphylococcus. The recombinant cells of the invention may be obtained by any method allowing the introduction of a foreign nucleotide sequence into a cell. This may be especially transformation, electroporation, conjugation, fusion of protoplasts, or any other technique known to persons skilled in the art.

The subject of the present invention is also a process for the preparation of polypeptides as claimed from the culture of one of these recombinant cells. The polypeptide(s) thus obtained are recovered according to conventional methods after the culture.

The invention also relates to an isolated topoisomerase IV capable of being obtained from the expr ssion of all or part of th grlA gene (SEQ ID No. 2) and of all or part of the grlB gene (SEQ ID No. 3) or of th ir r sp ctiv d rivativ s.

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sequences hybridizing with all or part of the grlA or grlB gene and encoding a subunit of a topoisomerase IV as well as all the sequences derived from a degeneracy of the genetic code of these hybrid sequences or of the sequences corresponding to all or part of the grlA or grlB gene.

More preferably, it is an isolated topoisomerase IV derived from the expression of all or part of the grlA gene (SEQ ID No. 2) or of all or part of the grlB gene (SEQ ID No. 3).

The present invention relates more particularly to any topoisomerase IV behaving as a primary target towards fluoroquinolones.

According to a specific mode of the invention, it is topoisomerase IV of Staphylococcus aureus.

The claimed topoisomerase IV according to the invention is most particularly useful for targeting biologically active products such as for example potential antibiotics and especially molecules of the fluoroquinolone family. Advantageously, it may also be used to assay and/or identify products inhibiting the ATP-dependent DNA relaxing reaction and/or the products inhibiting the reaction of decatenation of catenanes of DNA.

The applicant has thus developed an assay for enzymatic activity which is specific for topoisomeras IV of S. aureus and has shown that this activity is

inhibited by antibiotic molecules such as fluoroquinolones.

The present invention provides a new target for searching for new antibiotics, as well as a screen specific for this target; this screen is described in Example 7. This screen makes it possible to demonstrate the products which inhibit DNA topoisomerase IV of S. aureus. The following may be used in this test: pure or mixed synthetic products, natural plant extracts, bacterial cultures, fungi, yeasts or algae, pure or in the form of a mixture. The test described in the present invention makes it possible to detect both products which stabilize the cleavable complex, a reaction intermediate of the reaction catalysed by the enzyme, and also inhibitors acting through other mechanisms.

The examples and figures presented below by way of nonlimiting illustration show other advantages and characteristics of the present invention.

LEGEND TO THE FIGURES

Figure 1: Restriction map of the 4565 bp fragment containing the grlB and grlA genes of S. aureus.

Figure 2: Construction of the plasmids for expression of grlA and grlB. The constructs produced with grlA are schematically represented in A and those of grlB are in B. The cloned S. aureus DNA is represented by the

shaded rectangles, th vectors d rived from M13 ar in a thick black lin and th expr ssion vectors ar in a fine black line, the SstI restriction site is in brackets because it is a cloning site.

Figure 3: PAGE-SDS electrophoresis gel stained with Coomasie blue. Total cell extracts are deposited, lanes: 1 and 2, XL1-blue, pXL2340; 3 and 4, XL1-blue, pRSETB; 5 and 6, XL1-blue, pXL2320. The molecular weight markers (in hundreds) are indicated on the right of the figure. The arrow shows the overproduced protein. The + or - signs represent the induction with or without IPTG.

Figure 4: ATP-dependent relaxation activity of the GrlAB protein. The control experiments with purified DNA topoisomerase IV of E. coli (Peng and Marians, 1993) and purified DNA gyrase of E. coli (Hallet et al., 1990) are also described.

Figure 5: Decatenation activity of the protein GrlAB. kDNA, kinetoplast DNA; monomers, relaxed and decatenated DNA monomers. TopoIV: purified DNA topoisomerase IV of E. coli (50 ng); Gyrase: purified DNA gyrase of E. coli (50 ng); GrlA: GrlA protein extract (2 μg); GrlB: GrlB protein extract (2 μg); GrlAB: GrlA protein extract (2 μg) mixed with the GrlB protein xtract (2 μg).

Example 1 - PCR amplification of DNA fragments of Staphylococcus aureus which are inside the grlA and grlB genes.

This example describes the production of DNA fragments of Staphylococcus aureus which are inside the grlA and grlB genes. These fragments were obtained after PCR amplification carried out at 50°C with genomic DNA of the Staphylococcus aureus strain RN4220 (Novick, 1990) and of the degenerate oligonucleotides corresponding to the amino acids conserved in the N-terminal regions of the subunits GyrA of E. coli and B. subtilis and ParC of E. coli or of the subunits GyrB of E. coli and B. subtilis and ParE of E. coli. More specifically, the sense oligonucleotides 2137 and antisense oligonucleotides 2135 made it possible to amplify fragments of 255 bp which can encode 85 amino acids which would correspond to positions 39 to 124 on the E. coli GyrA sequence; the sequence of the sense oligonucleotide 2137 is 5'-GCGCGAATTCGATGG(A,T)(C,T)T-(A,T)AAACC(A,T)GT(A,T)CA-3' (SEQ ID No. 4) and that of the antisense 2135 is 5'-CGCGAAGCTTTTC(T,A)GTATA(A,T)C-(T,G)CAT(A,T)GC(A,T)GC-3' (SEQ ID No. 5). The oligonucleotides 2144 and 2138 led to the amplification of 1 kb fragments which can encode 333 amino acids which would correspond to positions 98 to 430 on the E. coli GyrB sequ nc ; the sequence of the sens oligonucl otide 2144 is 5'-GCGCGAATTCT(T,A)CATGC(A,T)-GG(T,A)GG(T,A)AAATT-3' (SEQ ID No. 6), and that of th

antisense 2138 is 5'-CGCGAAGCTT(T,A)CC(T,A)CC(T,A)GC-(T,A)GAATC(T,A)CCTTC-3' (SEQ ID No. 7) . The fragments were cloned and a total of 40 clones were analysed by sequencing their insert. The sequence of the oligonucleotides used for the PCR was found for 31 clones out of 40; among the 31 clones, 20 have a sequence which is inside the gyrA or gyrB gene of S. aureus; the other 11 clones contain either a fragment A of 255 bp or a fragment B of 1 kb.

The amino acid sequence which the A fragment is thought to encode has 59% identity with the GyrA subunit of S. aureus between positions 44 to 125, the A fragment is therefore thought to be part of an S. aureus grlA gene thus newly identified. Likewise, the amino acid sequence which the B fragment is thought to encode has 51% identity with the GyrB subunit of S. aureus between positions 105 to 277, the B fragment is therefore thought to be part of an S. aureus grlB gene thus newly identified.

Example 2 - Cloning and sequencing of the grlA and grlB genes of Staphylococcus aureus.

This example describes the molecular biology experiments which have made it possible to clone and then sequence the grlA and grlB genes of Staphylococcus aureus.

The A and B fragments described in Example 1 were used as radioactive probe to identify, by

hybridization, the grlA and grlB genes in a genomic DNA library of S. aureus FDA 574 (CE nt$^+$) constructed in λgt11 by Clontech Laboratories (catalogue XL1501b, batch 0721). Out of a total of 250,000 recombinant phages, twelve phages hybridize with the A fragment or the B fragment but do not hybridize with oligonucleotides specific for the gyrA or gyrB genes. The size of the EcoRI inserts contained in these phages varies between 0.7 and 3.5 kb and two phages, 16 and 111, whose insert is of a larger size, were studied. The EcoR1 insert of 3.5 kb of the phage 16 was eluted and then digested with XbaI and the two fragments of 1.5 and 2 kb were cloned into M13mp19 and M13mp18 (Boehringer Mannheim) in order to generate pXL2321 and pXL2322. Likewise, the EcoRI insert of 3.6 kb of the phase 111 was eluted and then digested with PstI and the 2 kb fragment was cloned into M13mp19 in order to generate pXL2324.

The inserts contained in the recombinant phages pXL2321, pXL2322 and pXL2324 were sequenced on both strands with the aid of the universal primer or of internal oligonucleotides using the Sanger method. The nucleic sequence grlAB (SEQ ID No. 1) of 4565 bp was analysed with the programme by Staden et al., 1982 in order to identify the coding sequences with the aid of a codon usage table for S. aureus. Only two open frames ORF1 (positions 41 to 2029) and ORF2 (positions 2032 to 4431) were thus determined. On SEQ ID No. 1, the coding

strand is th 5'->3' top strand, the open fram ORF1 starts arbitrarily at ATG position 41 but it can also start at TTG position 17 or 35, this codon being already described as initiation codon in S. aureus; the stop codon of ORF1 overlaps with the initiation codon GTG of ORF2, which is characteristic of a translational coupling (Normark et al., 1983); such a coupling has, for example, been described for the gyrA and gyrB genes of Haloferax sp. (Holmes et al., 1991). These open frames have a percentage of GC of 34.5% which is a value in agreement with the values described for the S. aureus DNA in the literature (Novick, 1990). Moreover, the B fragment is identical to the sequence described on SEQ ID No. 1 from position 333 to position 1348 in ORF1 and the fragment A is identical to the sequence of SEQ ID No. 1 from position 2137 to position 2394 in ORF2. From the nucleotide sequence, a restriction map is produced with enzymes which cut least frequently, see Figure 1.

This sequence analysis shows that ORF1 is the grlB gene and ORF2 the grlA gene.

Example 3 - Primary structure, expression and function of the GrlA and GrlB proteins encoded by the grlA and grlB genes of Staphylococcus aureus.

This xampl d scrib s the primary structur , th xpr ssion in E. coli and the function of th GrlA and GrlB prot ins of Staphylococcus aureus. This

function, which corresponds to a DNA topoisom ras IV, is based, in this example, on sequence homology and genetic complementation data.

3.1 - Primary structure and sequence analysis of the GrlA and GrlB proteins.

This example describes computer analysis of the sequence of the grlA and grlB genes of Staphylococcus aureus carried out using the sequence data presented in Example 2. The grlB gene encodes a GrlB protein of 663 amino acids (molecular weight 74,318), and the grlA gene encodes a GrlA protein of 800 amino acids (molecular weight 91,040). The coding parts of the grlB and grlA genes, the sequences of the GrlB and GrlA proteins are presented in SEQ ID No. 3 and SEQ ID No. 2 respectively and the properties of each of these proteins (amino acid composition, isoelectric point, polarity index) are presented in Tables 1 and 2 below.

Protein: GrlA:

First residue = 1 and last residue = 800

Molecular mass (monoisotopic) = 91040.8438

Molecular mass (average) = 91097.2578

Polarity ind x (%) = 52.00

Iso lectric point = 6.77

OD 260 (1 mg/ml) = 0.298 OD 280 (1 mg/ml) = 0.487

			NUMBER	% NOMB	WEIGHT	%WEIGHT
1	Phe	F	22	2.75	3235.51	3.55
2	Leu	L	74	9.25	8368.22	9.19
3	Ile	I	77	9.63	8707.47	9.56
4	Met	M	19	2.38	2489.77	2.73
5	Val	V	59	7.38	5845.04	6.42
6	Ser	S	51	6.38	4438.63	4.88
7	Pro	P	22	2.75	2135.16	2.35
8	Thr	T	43	5.38	4345.05	4.77
9	Ala	A	37	4.63	2628.37	2.89
10	Tyr	Y	28	3.50	4565.77	5.02
12	His	H	20	2.50	2741.18	3.01
13	Gln	Q	26	3.25	3329.52	3.66
14	sn	N	45	5.63	5131.93	5.64
15	Lys	K	66	8.25	8454.27	9.29
16	Asp	D	54	6.75	6211.45	6.82
17	Glu	E	67	8.38	8645.85	9.50
18	Cys	C	0	0.00	0.00	0.00
19	Trp	W	2	0.25	372.16	0.41
20	Arg	R	44	5.50	6868.45	7.54
21	Gly	G	44	5.50	2508.94	2.76

TABLE 1

GrlB protein:

First residue = 1 and last residue = 663

Molecular mass (monoisotropic) = 74318.3516

Molecular mass (average) = 74363.9219

Polarity ind x (%) = 53.70

Iso l ctric point = 7.21

OD 260 (1 mg/ml) = 0.404 OD 280 (1 mg/ml) = 0.603

			NUMBER	% NOMB	WEIGHT	%WEIGHT
1	Phe	F	26	3.92	3823.78	5.15
2	Leu	L	55	8.30	6219.62	8.37
3	Ile	I	36	5.43	4071.03	5.48
4	Met	M	10	1.51	1310.40	1.76
5	Val	V	50	7.54	4953.42	6.67
6	Ser	S	41	6.18	3568.31	4.80
7	Pro	P	15	2.26	1455.79	1.96
8	Thr	T	41	6.18	4142.95	5.57
9	Ala	A	33	4.98	2344.22	3.15
10	Tyr	Y	19	2.87	3098.20	4.17
12	His	H	14	2.11	1918.82	2.58
13	Gln	Q	26	3.92	3329.52	4.48
14	Asn	N	36	5.43	4105.55	5.52
15	Lys	K	63	9.50	8069.98	10.86
16	Asp	D	40	6.03	4601.08	6.19
17	Glu	E	61	9.20	7871.60	10.59
18	Cys	C	0	0.00	0.00	0.00
19	Trp	W	4	0.60	744.32	1.00
20	Arg	R	34	5.13	5307.44	7.14
21	Gly	G	59	8.90	3364.27	4.53

TABLE 2

The Kanehisa programme, described in 1984, was used to align the GrlB and GrlA proteins with the following type II bacterial DNA topoisomerases, the *E. coli*, *B. subtilis* or *S. aureus* gyrases or the *E. coli* topoisomerase IV. The degrees of identity, see Table 3, are high and are between 32 and 55%. More specifically, GrlB exhibits a greater degree of identity with the GyrB subunits of *E. coli* (49%) and of

S. aureus (52%) than with ParE of E. coli (38%), whereas GrlA exhibits comparable degrees of identity with the GyrA subunits of E. coli (32%) and of S. aureus (39%) than with ParE of E. coli (33%).

The GyrB subunits of Staphylococcus aureus (Margerrison et al., 1992), Bacillus subtilis (Moriya et al., 1985), and Escherichia coli (Adachi et al., 1987) are called SAGYRB, BSGYRB and ECGYRB respectively, GrlB is called SAGRLB and ECPARE corresponds to ParE of E. coli (Kato et al., 1990). A similar nomenclature is used for the GyrA, GrlA and ParC subunits. The numbers under the name of the proteins are the numbers of amino acids in them.

B or B-like subunits	SAGYRB 644	SAGRLB 663	BSGYRB 638	ECGYRB 804
SAGRLB	52%			
BSGYRB	68%	55%		
ECGYRB	55%	49%	57%	
ECPARE	40%	38%	40%	40%
A or A-like subunits	**SAGYRA 887**	**SAGRLA 800**	**BSGYRA 821**	**ECGYRA 875**
SAGRLA	39%			
BSGYRA	65%	40%		
ECGYRA	39%	32%	41%	
ECPARC	38%	33%	36%	32%

TABLE 3

Multipl alignments b tween the typ II bacterial topoisom rases, p rformed with the CLUSTAL programme of Higgins t al., 1988, show numerous

conserved regions between the sequences of the various B, GrlB and ParE subunits and in the N-terminal part of the sequence of the A, GrlA and ParC subunits. The residues conserved in the N-terminal region of the B subunits of these proteins are in fact the residues involved in the binding of ATP and identified from X-ray crystallization data with the E. coli GyrB (Wigley et al., 1991). The residues conserved in the N-terminal region of the A subunits of these proteins are either the residues AAMRYTE (SEQ ID No. 8) close to the active site of gyrase Tyr-122, identified on the E. coli GyrA (Horowitz et al., 1987), or the residues YHPHGDS (SEQ ID No. 9) modified in the strains resistant to fluoroquinolones (Hooper et al., 1993).

3.2 - Expression of the grlA and grlB genes in E. coli.

This example describes the constructs produced in order to express, in E. coli, the grlA or grlB genes under the control of the pT7 promoter (Studier et al., 1990). The expression plasmid pXL2320, see Figure 2, containing the grlB gene in the vector pRSETB (Studier et al., 1990; Invitrogen) was constructed by cloning 1) the 1 kb EcoRI-XbaI insert of pXL2321 into pXL2322 at the XbaI and EcoRI sites in order to gen rat pXL2323; 2) the 1.9 kb KpnI-EcoRI insert of pXL2323 at the KpnI and EcoRI sites of the vector pRSETB in order to gen rat pXL2319; the 0.5 kb NdeI-KpnI insert of pXL2325 at the NdeI and KpnI sites

of pXL2319 in order to obtain pXL2320. (pXL2325 contains the first 500 bases of the gene where a CAT sequence has been introduced by mutagenesis, just upstream of the ATG initiation codon, in order to create an NdeI site). The grlB gene expression cassette contained in pXL2320 was cloned at the BglII and EcoRI sites of pKT230 (Bagdasarian et al., 1981) in order to obtain pXL2439. The expression plasmid pXL2340, see Figure 2, containing the grlA gene in the vector pRSETB was constructed by cloning 1) the 1.7 kb NdeI-EcoRI insert of pXL2324 at the NdeI and EcoRI sites of the vector pRSETB in order to generate pXL2338; the 0.75 kb NdeI insert of pXL2337 at the NdeI sites of pXL2338 in order to obtain pXL2340. (pXL2337 contains the first 750 bases of the gene where a CATATG sequence has been introduced by mutagenesis, in place of the GTG initiation codon in order to create an NdeI site).

The plasmids pXL2320, or pXL2340 were introduced into the E. coli XL-1-Blue strain (Stratagen) and the expression of the genes was induced when the T7 phage RNA polymerase was produced after induction of the gene, encoding the T7 phage RNA polymerase, cloned into the helper phage M13/T7 (Studier et al., 1990, Invitrogen). The cellular extracts were analysed by electrophoresis on a PAGE-SDS gel stained with Coomasie blue as has already been described (Denèfle et al., 1987). In Figure 3 is represented the production of a protein with a

i) molecular weight of 79,000, when the grlB gene is induced in the E. coli strain XL1-blue, pXL2320; and ii) molecular weight of 90,000, when the grlA gene is induced in the E. coli strain XL1-Blue, pXL2340. The measured molecular weights are in agreement with the molecular weights deduced from the sequence.

3.3 - Complementation of the parCts and parEts mutants of Salmonella typhimurium by the grlA and grlB genes of Staphylococcus aureus.

This example describes the heterologous complementation of the S. typhimurium parCts and parEts mutants by the S. aureus grlA and grlB genes. The plasmids pXL2320, pXL2340, pXL2439 or the vector pRSETB were introduced into the S. typhimurium strains SE7784 (parC281(Ts) zqe-2393::Tn10 leu485) or SE8041 (parE206(Ts) zqe-2393::Tn10 leu485) (Luttinger et al., 1991). No plasmid complements the heat-sensitive phenotype; on the other hand, when the plasmids pXL2340 and pXL2439 are introduced simultaneously into the SE7784 strain or into the SE8041 strain, the heat-sensitive phenotype of both strains is complemented. Consequently, the coexpression of the grlA and grlB genes of S. aureus allows the complementation of the ParC Ts or ParE Ts phenotype of the S. typhimurium mutants.

Example 4 - The DNA topoisomerase IV of S. aureus is the primary target of the fluoroquinolones.

This example describes the presence of a point mutation Ser-80 in the GrlA subunit with all the analysed clinical strains of S. aureus resistant to the fluoroquinolones whereas a mutation in the QRDR region (Quinolone Determining Region) (equivalent to the Ser-80 region of GrlA) in the GyrA subunit does not exist with the clinical strains of S. aureus weakly resistant to the fluoroquinolones. Consequently, the GrlA subunit is shown to be the primary target of the fluoroquinolones in S. aureus.

The genomic DNA of eight clinical strains of S. aureus and of a laboratory strain was prepared and used to amplify at 42°C by PCR: i) the first 500 base pairs of gyrA using the sense oligonucleotide 5'-GGCGGATCCCATATGGCTGAATTACCTCA-3' (SEQ ID No. 10) and the antisense oligonucleotide 5'-GGCGGAAT TCGACGGC-TCTCTTTCATTAC-3' (SEQ ID No. 11); ii) and the first 800 base pairs of grlA using the sense oligonucleotide 5'-GGCCGGATCCCATATGAGTGAAATAATTCAAGATT-3' (SEQ ID No. 12) and the antisense oligonucleotide -5'-GGCCGAATTCTAATAATTAACTGTTTACGTCC-3' (SEQ ID No. 13). Each amplified fragment was cloned into the phage M13mp18 and th s qu nc of th first 300 base pairs of ach of th g nes was read n 2 clones. The gyrA sequence is identical to that published by Magerrison and that of grlA to that d scrib d in SEQ ID

No. 1, with th exception of th mutations pres nted in Table 4. The mutations in gyrA exist with the strains highly resistant to fluoroquinolones (SA4, SA5, SA6, SA35, SA42R and SA47; MIC for ciprofloxacin > 16 mg/l); these mutations are a base change which leads to changes in the amino acids Ser-84 or Ser-85 or Glu-88. A mutation in grlA exists with all the strains resistant to fluoroquinolones and corresponds to the changing of the residue Ser-80 to Phe or Tyr.

Strain	MIC mg/l Ciprofloxacin	Mutation in *gyrA*		Mutation in *grlA*	
		Base	Codon	Base	Codon
RN4220*	1	no	no	no	no
SA42*	0.5	no	no	no	no
SAH**	2	no	no	2281 G->A	^{84}Glu->Lys
SA1*	2	no	no	2270 C->T	^{80}Ser->Phe
SAA**	4	no	no	2281 G->A	^{84}Glu->-Lys
SA3**	4	no	no	2270 C->T	^{80}Ser->Phe
SA2**	16	no	no	2270 C->A	^{80}Ser->Tyr
SA47*	16	2533 C->T*	^{84}Ser->Leu	2270 C->A	^{80}Ser->Tyr
SA4**	32	2544 G->A	^{88}Glu->Lys	2270 C->T	^{80}Ser->Phe
SA5**	32	2533 C->T	^{84}Ser->Leu	2270 C->T	^{80}Ser->Phe
SA6**	32	2533 C->T	^{84}Ser->Leu	2270 C->T	^{80}Ser->Phe
SA35*	64	2535 T->C	^{85}Ser->Pro	2270 C->A	^{80}Ser->Tyr
SA42R*	>128	2533 C->T*	^{84}Ser->Leu	2270 C->A	^{80}Ser->Tyr

TABLE 4

* already published by Sreedharan et al. (1990)

** strains obtained from French state hospitals.

Example 5 - PCR (Polymerase Chain Reaction) amplification of the S. aureus DNA fragment which is inside grlA containing a point mutation which leads to a substitution in GrlA from Ser-80 to Tyr (Ser-80→Tyr).

This example describes the production of the DNA fragment which is inside grlA of an S. aureus strain, SA2, resistant to fluoroquinolones. The grlA

fragment contains a base change from C to A at position 2270 of the wild-type gene (Fig. 1). This mutation leads to a substitution of the residue Ser-80 to Tyr in the GrlA protein. It has been shown that a substitution of the residue Ser-80 to Phe or Tyr exists with all the strains weakly resistant to fluoroquinolones (Example 4). The fragment which is inside grlA was obtained after PCR amplification carried out at 50°C with the genomic DNA of the SA2 strain and of the oligonucleotides 3358 and 3357 which correspond to position 2036 and 3435 respectively on the sequence of grlA. More specifically, the sense oligonucleotide 3358 (SEQ ID No. 12) (Example 4) and the antisense oligonucleotide 3357 made it possible to amplify a fragment of 1399 base pairs; the sequence of the antisense oligonucleotide 3357 is 5'-GGCCGAGCTCC-AATTCTTCTTTTATGACATTC-3' (SEQ ID No. 14). The oligonucleotide 3358 was also used to introduce, by mutagenesis, a sequence CATATG, in place of the GTG initiation codon in order to create an NdeI site. The amplified grlA fragment was cloned into the BamHI/SstI cloning sites of pUC18 (Boehringer Mannheim), and 6 clones containing this plasmid, pXL2692, were analysed after sequencing their insert. In all cases, a sequence CATATG was introduced in place of the CTG initiation codon, and the point mutation at position 2270 of grlA (C→A) was again found.

Example 6 - Expression in *E. coli* of the *grlA* gene containing a base change corr sponding to th change of the residue Ser-80 to Tyr.

This example describes the construct prepared in order to express, in *E. coli*, the mutated *grlA* gene under the control of the T7 promoter (Studier et al., 1990). The expression plasmid pXL2742, containing the mutated *grlA* gene, was constructed by cloning the 0.75 kb insert of pXL2692 into the *Nde*I site of pXL2338 (Example 3.2). The plasmid pXL2742 was introduced into the *E. coli* XL1-Blue strain and the expression of the *grlA* gene was carried out as described in Example 3.2. The production of a protein having a molecular weight of 90,000 was obtained with the plasmid pXL2742 containing the *grlA* gene. The molecular weight measured is in agreement with the molecular weight deduced from the sequence of the *grlA* gene, and that already obtained for the wild-type GrlA protein (Example 3.2).

Example 7 - DNA topoisomerase IV activity of the GrlAB protein of *S. aureus*.

This example illustrates how an acellular extract containing the GrlAB protein can be prepared and how the enzymatic activity of the GrlAB protein present in this extract can be detected and measured.

7.1 - Pr paration of the cell extracts.

An acellular extract of th *E. coli* strain

XL1-blue pXL2340 expressing the Grl A prot in is pr pared for example in the following manner:

The E. coli strain XL1-blue pXL2340 is cultured as follows: 250 ml of LB medium containing ampicillin at 50 mg/l are inoculated at 1/100 with a culture of E. coli XL1-blue pXL2340, and incubated at 30°C; when the optical density at 600 nm is 0.3, 1 mM IPTG is added; after incubating for 30 min at 37°C, the strain is infected with the helper phage M13/T7 with a multiplicity of infection of 5 pfu per cell for 4 hours. After centrifugation (5000 × g; 20 min), the cells obtained using 1.5 litres of culture are resuspended in 20 ml of 50 mM Tris/HCl buffer pH 7.8 containing 10 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.12% Brij 58 and 0.75 mg/ml of lysozyme. After 30 min at 4°C, the mixture is centrifuged for 1 h at 50,000 × g and the supernatant containing the Grl A protein is recovered. A change of buffer is carried out on this sample by chromatographing the extract through a column filled with Sephadex G625 (Pharmacia) equilibrated and eluted with the 50 mM Tris/HCl buffer pH 7.5 containing 1 mM EDTA, 5 mM DTT, 100 mM NaCl and 10% sucrose.

An acellular extract containing the Grl B protein is prepared in a similar manner using the E. coli strain XL1-blue pXL2320.

7.2 - Purification of the DNA topoisom rase IV of S. aur us.

This example illustrates how an S. aureus enzyme catalysing the segregation of the daughter chromosomes during the final phase of replication (topoisomerase IV) can be purified.

The purification of the two GrlA and GrlB subunits of topoisomerase IV is carried out as described below, using the decatenation activity assay described in Example 7.3 to detect the presence of the GrlA and GrlB proteins during the purification, as is commonly used by persons skilled in the art. During the assay of this enzymatic activity, complementation of the fractions containing the GrlA protein is obtained with 1 µg of proteins of an extract of the E. coli strain XL1-blue pXL2320 expressing the GrlB subunit, and complementation of the fraction containing the GrlB protein is obtained with 1 µg of proteins of an extract of the E. coli strain XL1-blue pXL2340 expressing the GrlA subunit. A preferred mode of preparation of the enzymatic extracts is described in Example 7.1. Between each stage, the fractions containing the desired protein are frozen and stored at -70°C.

The purification of the A subunit may be carried out by chromatography, for example, according to th following proc dur :

an ac llular extract prepared as d scribed in Example 7.1 using about 5 g of c lls of E. coli XL1-blu

pXL2340 is chromatograph d on a MonoQ HR 10/10 column (Pharmacia) at a flow rate of 3 ml/min with a linear gradient of NaCl (0.1M to 0.6M over 60 min) in a 10 mM Tris/HCl buffer pH 8.0 containing 1 mM EDTA, 1 mM DTT and 10% glycerol (w/v). The active fractions are combined and the sample is chromatographed on a Superdex 200 HiLoad 26/60 column (Pharmacia) equilibrated and eluted with 50 mM Tris/HCl buffer pH 7.5 containing 1 mM EDTA, 5 mM DTT and 0.25 M NaCl. The GrlA protein, which exists in the form of a symmetrical peak, is coeluted with the desired activity. After this stage, the preparation shows a single visible band in SDS-PAGE after developing with silver nitrate, and this band migrates with an apparent molecular weight of about 90,000.

The purification of the B subunit may be carried out by chromatography, for example, according to the following procedure:

an acellular extract prepared as described in Example 5 using about 5 g of cells of E. coli XL1-blue pXL2320 is injected onto a Novobiocin-Sepharose CL-6B column (6 ml of gel prepared according to the procedure described by Staudenbauer et al., 1981, Nucleic Acids Research) equilibrated in 50 mM Tris/HCl buffer pH 7.5 containing 1 mM EDTA, 5 mM DTT and 0.3 M NaCl. After washing th column with the same buffer, the GrlB protein is elut d with 50 mM Tris/HCl buffer pH 7.5 containing 1 mM EDTA, 5 mM DTT and 2 M NaCl and 5 M ur a. This fraction is

then chromatographed on a Superdex 200 HiLoad 26/60 gel permeation column (Pharmacia) equilibrated and elut d with 50 mM Tris/HCl buffer pH 7.5 containing 1 mM EDTA, 5 mM DTT and 0.25 M NaCl. The GrlB protein, which exists in the form of a symmetrical peak, is coeluted with the desired activity. After this stage, the preparation has a single visible band in SDS-PAGE after developing with silver nitrate, and this band migrates with an apparent molecular weight of about 80,000.

7.3 - Detection of the enzymatic activities of the GrlAB protein.

The various enzymatic activities of the GrlAB protein are detected by incubating, in the same reaction mixture, equal quantities of the two types of extracts prepared by the process described above or by any other process which makes it possible to recover the intracellular enzymatic proteins of the microorganism while preserving their activity, such as for example the procedures involving the use of presses (such as the French Press, the X-Press), or the use of ultrasound.

The ATP-dependent supercoiled DNA relaxing activity can be detected by carrying out the procedure, for example, in the following manner:

a mixture of an xtract of th <u>E. coli</u> strain XL1-blu pXL2320 (1 µg of proteins) and of an xtract of the <u>E. coli</u> strain XL1-blu pXL2340 (1 µg of prot ins) is

incubated for 1 h at 37°C in 30 µl of 50 mM Tris/HCl buffer pH 7.7 containing 4 mM ATP, 6 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 20 mM KCl, 50 µg/ml of bovine serum albumin and 500 ng of supercoiled plasmid pBR322. The reaction is stopped by adding 7 µl of a 5% SDS and 2.5 mg/ml proteinase K mixture and the samples are incubated for a second period of 30 min at 37°C and then analysed by electrophoresis on 1% agarose gel in 0.1M Tris/borate buffer pH 8.3 containing 2 mM EDTA at 6V/cm for 3 h. The separation of the relaxed and nicked (open circular) DNAs is carried out by performing an additional 2 h electrophoretic run after addition of ethidium bromide (1 µg/ml) to the running buffer. The DNA is then quantified by scanning the negatives of photographs of the gels (Polaroid type 665 film) with the aid of a Bioimage 50S apparatus (Millipore).

Figure 4 shows that the acellular extracts of the strains E. coli XL1-blue pXL2320 and E. coli XL1-blue pXL2340 exhibit in a mixture an intense DNA relaxing activity whereas each of the extracts is inactive when it is incubated alone. The reaction is ATP-dependent. Furthermore, these two extracts, alone or in the form of a mixture, exhibit no DNA supercoiling activity, an activity typical of gyrase.

Th ATP-dep nd nt activity of decat nation of intertwin d circular DNA molecul s (cat nan s) can be detected by carrying out the proc dur , for exampl , in the following manner:

a mixtur of an extract of the E. coli strain XL1-blu pXL2320 (2.5 μg of prot ins) and of an xtract of th E. coli strain XL1-blue pXL2340 (2.5 μg of proteins) is incubated for 1 h at 37°C in 40 μl of 50 mM Tris/HCl buffer pH 7.7 containing 1 mM ATP, 6 mM MgCl2, 200 mM glutamate, 10 mM DTT, 10 mM NaCl, 50 μg/ml of bovine serum albumin and 800 ng of kinetoplast DNA [consisting of a network of intertwined DNA molecules (catenanes) obtained from Crithidia fasciculata; TopoGene]. The reaction is stopped by adding 7 μl of a 250 mM EDTA solution (incubation 5 min at 37°C), 5 μl of a 5% SDS and 2.5 mg/ml proteinase K mixture (incubation 30 min at 37°C). The mixture is then analysed by electrophoresis on a 1% agarose gel in a 0.1M Tris/borate buffer pH 8.3 containing 2 mM EDTA at 6V/cm for 2 h 30 min. After staining the DNA with ethidium bromide (1 μg/ml), the DNA is quantified by scanning the negatives of photographs of the gels (Polaroid type 665 film) with the aid of a Bioimage 50S apparatus (Millipore). By working, for example, under the conditions described above, the extracts of the two strains E. coli XL1-blue pXL2320 and E. coli XL1-blue pXL2340 exhibit, in the form of a mixture, an activity of complete decatenation of the starting kinetoplast DNA. This activity is detected by the appearance of a DNA band with a size of about 2.5 kb and by the disappearance of the band of catenated DNA of very large size which penetrates very little into the gel

during the electrophoretic run (Figur 5). The *E. coli* gyrase introduc d as a control into this assay exhibits no decatenation activity contrary to DNA topoisomerase IV of *E. coli* which completely decatenates the kinetoplast DNA (Figure 5).

Example 8 - DNA topoisomerase IV activity of the GrlAB protein of *S. aureus* whose GrlA subunit exhibits a substitution of the residue Ser-80 to Tyr (Ser-80→Tyr).

8.1 - Preparation of a cell extract containing the GrlAB protein of *S. aureus* whose GrlA subunit exhibits a substitution of the residue Ser-80 to Tyr (Ser-80→Tyr).

This example illustrates how an acellular extract containing the protein GrlA$_{(Ser\text{-}80\rightarrow Tyr)}$B can be prepared, and how the enzymatic activity of the protein GrlA$_{(Ser\text{-}80\rightarrow Tyr)}$B can be detected and measured.

An acellular extract of the *E. coli* strain XL1-Blue pXL2742 expressing the protein GrlA$_{(Ser\text{-}80\rightarrow Tyr)}$ is prepared, for example, as described in Example 7 for the wild-type GrlA protein.

8.2 - Purification of a DNA topoisomerase IV of *S. aureus* exhibiting an Ser-80-Tyr mutation in the GrlA subunit.

This example illustrates how a topoisomerase IV of *S. aureus* exhibiting an Ser-80→Tyr mutation in

the GrlA subunit can be purified. The GrlA subunit of topoisomerase IV having an Ser-80→Tyr mutation is purifi d according to a procedure identical to that described in Example 7.2 using a culture of the E. coli strain XL1-blue pXL2742 constructed as described in Example 6.

8.3 - Detection of the enzymatic activities.

The ATP-dependent activities of supercoiled DNA relaxation, on the one hand, and of decatenation of intertwined circular DNA molecules, on the other hand, are detected in this extract as described in Example 7, by incubating, in the same reaction mixture, an acellular extract of the E. coli strain XL1-Blue pXL2742 containing the protein GrlA(Ser-80→Tyr) and an extract of the E. coli strain XL1-Blue pXL2320 containing the GrlB protein.

Example 9 - Inhibition by fluoroquinolones, of the DNA topoisomerase IV activity of the wild-type GrlAB protein of S. aureus and resistance to fluoroquinolones of the protein containing an Ser-80→Tyr transition in the GrlA subunit.

The two methods described in Example 7 for the assay of DNA topoisomerase IV activities can be us d to detect novel molecul s which act as inhibitors of topoisom rase IV of S. aureus or to characterize the behaviour of topoisomerase IV of S. aureus towards

molecules already id ntified as inhibitors of oth r topoisomerases (for exampl the fluoroquinolones).

In the test of relaxation of supercoiled DNA for example, the disappearance or the decrease in the relaxed DNA band during analysis of the reaction mixture after incubation of the GrlAB protein of S. aureus in the presence of a molecule or of a mixture of several molecules indicates that this molecule (or these molecules), inhibit the relaxation activity of GrlAB, and is therefore potentially antibacterial. However, since the studies carried out up until now (described in Example 7) have demonstrated that the GrlAB protein is a topoisomerase IV, and since it is nowadays established that the major function of the topoisomerases IV is decatenation (or disentanglement) of the intertwined daughter chromosomes during the final stages of replication, it seems more judicious to search for the inhibitors of the GrlAB protein using a test of decatenation of DNA using, for example, the test described in Example 7.3. To carry out the experiments described in the examples which follow, the incubations are carried out with the purified wild-type GrlAB proteins as described in Example 7, and with the mutant protein GrlA(Ser-80→Tyr)B as described in Example 8. The two wild-type and mutant GrlAB proteins are reconstituted by mixing equimolar quantities of their two GrlA and GrlB subunits.

In th d cat nation t st, if the disappearance or th

decrease in the intensity of the decatenated DNA band is observ d during analysis of the reaction mixture after incubation of the GrlAB protein in the presence of a molecule or of a mixture of several molecules, this indicates that this molecule (or these molecules) inhibits the decatenation activity of the GrlAB protein, and is therefore potentially antibacterial. Since it has been demonstrated in the present invention that the GrlAB protein is the primary target for the molecules of the fluoroquinolone family, it appears that the fluoroquinolones must act as inhibitors in the decatenation test described in Example 7. Indeed, when the purified GrlAB protein is incubated in the presence of increasing quantities of a fluoroquinolone, for example ciprofloxacin, it appears that above a concentration of 10 μg/ml, ciprofloxacin completely inhibits the activity of decatenation of the kinetoplast DNA. Ciprofloxacin inhibits 50% of the activity of decatenation of kinetoplast DNA at a concentration of 4.0 μg/ml.

Likewise, sparfloxacin which is another fluoroquinolone inhibits 50% of the activity of decatenation of kinetoplast DNA at a concentration of 6.0 μg/ml.

Likewise, since it has been demonstrated in the present invention (Example 4) that the presence of an Ser-80→Tyr mutation on th GrlA subunit of th mutant GrlAB protein conf rs on th strain a c rtain level of resistance to fluoroquinolones, for example

ciprofloxacin, it appears that the fluoroquinolones must act on this mutant DNA topoisomeras IV as inhibitors which are less efficient in the decatenation test described in Example 7.

Indeed, when the mutant protein GrlA(Ser-80→Tyr)B is incubated in the presence of increasing quantities of a fluoroquinolone, for example ciprofloxacin, it appears that ciprofloxacin inhibits 50% of the activity of decatenation of kinetoplast DNA at a concentration of 60 μg/ml, that is to say a concentration 15 times as high as that necessary to obtain the same effect with the wild-type enzyme.

Likewise, in the presence of the mutant enzyme GrlA(Ser-80→Tyr)B, sparfloxacin inhibits 50% of the activity of decatenation of kinetoplast DNA at a concentration of 500 μg/ml, that is to say a concentration 80 times as high as that necessary to obtain the same effect with the wild-type enzyme.

Norfloxacin inhibits 50% of the activity of decatenation of kinetoplast DNA at a concentration of 12 μg/ml with the wild-type GrlAB enzyme and exhibits the same inhibitory activity at a concentration of 125 μg/ml with the enzyme GrlA(Ser-80→Tyr)B. Ofloxacin inhibits 50% of the activity of decatenation of kin toplast DNA at a concentration of 10 μg/ml with the wild-typ GrlAB nzyme and has the sam inhibitory activity at a concentration of 250 μg/ml with the enzyme GrlA(Ser-80→Tyr)B.

Novobiocin, whos mechanism of action is different from that of the fluoroquinolones, should therefor in principle hav th same inhibitory activity on both the wild-type GrlAB enzyme and on the mutant GrlA(Ser-80→Tyr)B enzyme in the decatenation test described in Example 7. Indeed, novobiocin inhibits 50% of the activity of decatenation of kinetoplast DNA at a concentration of about 30 μg/ml whatever the enzyme used (wild-type GrlAB or mutant GrlA(Ser-80→Tyr)).

ABBREVIATIONS

DNA : deoxyribonucleic acid

RNA : ribonucleic acid

MIC : minimum inhibitory concentration

IPTG : isopropylthio-β-D-galactoside

LB : Luria-Bertani medium

PAGE : electrophoresis gel containing acrylamide and N,N'-methylenebisacrylamide

PCR : polymerase chain reaction

pfu : plaque forming unit

QRDR : region of the GyrA subunit where the point mutations leading to resistance to fluoroquinolones are mapped

SDS : sodium dodecyl sulphate

Tris : tris(hydroxymethyl)aminomethane

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